Divergent Targets of *Candida albicans* Biofilm Regulator Bcr1 *In Vitro* and *In Vivo*

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Candida albicans **is a causative agent of oropharyngeal candidiasis (OPC), a biofilm-like infection of the oral mucosa. Biofilm formation depends upon the** *C. albicans* **transcription factor Bcr1, and previous studies indicate that Bcr1 is required for OPC in a mouse model of infection. Here we have used a nanoString gene expression measurement platform to elucidate the role of Bcr1 in OPC-related gene expression. We chose for assays a panel of 134 genes that represent a range of morphogenetic and cell cycle functions as well as environmental and stress response pathways. We assayed gene expression in whole infected tongue samples. The results sketch a portrait of** *C. albicans* **gene expression in which numerous stress response pathways are activated during OPC. This one set of experiments identifies 64 new genes with significantly altered RNA levels during OPC, thus increasing substantially the number of known genes in this expression class. The** *bcr1Δ/Δ* **mutant had a much more limited gene expression defect during OPC infection than previously reported for** *in vitro* **growth conditions. Among major functional Bcr1 targets, we observed that** *ALS3* **was Bcr1 dependent** *in vivo* **while** *HWP1* **was not. We used null mutants and complemented strains to verify that Bcr1 and Hwp1 are required for OPC infection in this model. The role of Als3 is transient and mild, though significant. Our findings suggest that the versatility of** *C. albicans* **as a pathogen may reflect its ability to persist in the face of multiple stresses and underscore that transcriptional circuitry during infection may be distinct from that detailed during** *in vitro* **growth.**

Candida albicans is a major invasive fungal pathogen of humans and can cause both mucosal and disseminated infections. Infections of the oral mucosa in particular, called oropharyngeal candidiasis (OPC), affect HIV patients (41), diabetes patients (51), and head and neck cancer patients receiving radiation therapy (1, 39, 48). Our goal is to define the attributes of *C. albicans* that make it a successful oral pathogen.

Mucosal infections may be considered biofilms, in that the pathogen adheres to a surface and produces an extracellular matrix (15, 39). This analogy has prompted investigations that test the hypothesis that genes required for biofilm formation *in vitro* may be required for mucosal infection as well. Findings from these studies have underscored the utility of this perspective, in that there are several common genetic requirements for the formation of abiotic surface biofilms and mucosal infections (10, 11, 15, 19).

One of the central regulators of biofilm formation is the zinc finger transcription factor Bcr1. It was identified in screens for mutants defective in biofilm formation on abiotic surfaces (33–35) and in adherence to a silicone substrate (14). Bcr1-dependent genes have been defined under *in vitro* growth conditions (14, 33–35). Many are cell surface protein genes, including *ALS1*, *ALS3*, and *HWP1*. These three genes are major functional Bcr1 targets, in that they are required for abiotic surface biofilm formation, and their overexpression restores biofilm formation in *bcr1Δ/Δ* mutant backgrounds (33, 34).

Transcription factors like Bcr1 have long been used to define the functional basis of pathogenicity traits (8, 27, 42). One strength of this approach comes from the fact that virulence potential may arise from expression of gene families or other gene sets with overlapping functions. Because transcription factors often control functionally related target genes, a single transcription factor defect can abolish a function that is carried out by redundant genes. One weakness of this approach comes from the fact

that the spectrum of transcription factor target genes may be contingent upon environmental conditions. Thus, the gene expression impact of a transcription factor in an infection setting may be different from its impact *in vitro*. In fact, there are now several examples in which a target gene is expressed in colonization or infection samples independently of a transcriptional regulator that was defined by *in vitro* assays (24). Moreover, this limitation is not restricted to transcription factors, since almost any genetic perturbation has gene expression consequences (22) that may contribute to a mutant phenotype.

Clearly it is critical to assess pathogen gene expression *in vivo* during infection, and several prior studies have done so (2, 18, 21, 29, 36, 38, 47, 49, 52, 54). Relevant to oral *C. albicans* infection, there has been a quantitative reverse transcription (QRT)-PCR analysis of *C. albicans* gene expression in a gnotobiotic pig OPC model (50), which revealed that *ECE1* RNA accumulated at very high levels during infection. There have also been microarray analyses of both reconstituted human epithelial (RHE) infection and OPC patient samples (31, 54). The most highly upregulated genes in these contexts, compared to *in vitro* yeast extract-peptone dextrose (YPD)-grown cells, included the cell surface protein genes *ALS3* and *HWP1*. All three of these genes—*ECE1*, *ALS3*, and *HWP*—require Bcr1 for expression under *in vitro* growth conditions (35). These findings suggested that Bcr1 may be required for

TABLE 1 *Candida albicans* strains used in this study

Strain name	Genotype	Reference
BWP17	*ura3Δ::λimm434* *arg4::hisG* *his1::hisG*	53
	ura3Δ::λimm434 *arg4::hisG* *his1::hisG*	
DAY185	*ura3Δ::λimm434* *HIS1::his1::hisG* *ARG4::URA3::arg4::hisG*	53
	ura3Δ::λimm434 *his1::hisG* *arg4::hisG*	
CAI4-URA3	*ura3Δ::λimm434* *ARG4::pARG4-URA3*	40
	ura3Δ::λimm434 *ARG4*	
CJN698	*ura3Δ::λimm434* *arg4::hisG his1::hisG::pHIS1-BCR1* *bcr1:: ARG4*	35
	ura3Δ::λimm434 *arg4::hisG his1::hisG* *bcr1::URA3*	
CJN702	*ura3Δ::λimm434* *arg4::hisG his1::hisG::pHIS1* *bcr1::ARG4*	35
	ura3Δ::λimm434 *arg4::hisG his1::hisG* *bcr1::URA3*	
CAH7-1A1E2	*ura3Δ::λimm434* *hwp1::hisG* *eno1::URA3*	45
	ura3Δ::λimm434 *hwp1::hisG* *ENO1*	
CAHR3	*ura3Δ::λimm434* *hwp1::hisG* *eno1::URA3*	45
	ura3Δ::λimm434 *HWP1* *ENO1*	
CAYF178U	*ura3Δ::λimm434::URA3-IRO1* *als3::ARG4* *arg4::hisG* *his1::hisG*	33
	ura3Δ::λimm434 *als3::HIS1* *arg4::hisG his1::hisG*	
CAQTP178U	*ura3Δ::λimm434::URA3-IRO1* *als3::ARG4::ALS3* *arg4::hisG* *his1::hisG*	33
	ura3Δ::λimm434 *als3::HIS1* *arg4::hisG his1::hisG*	

OPC. Indeed, a recent analysis in a murine OPC model has shown that Bcr1 is required for infection (11). In that context, Bcr1 seemed to act through *HWP1*, because overexpression of *HWP1* significantly improved virulence of the *bcr1Δ/Δ* mutant. These findings were consistent with previous biochemical and functional analysis of Hwp1, which has shown that it is required for OPC in transgenic immunocompromised mouse models and that it is an epithelial adhesin (44, 45). Overexpression of *ALS3* also improved virulence by qualitative assay (11), though quantitative measures did not reach significance thresholds. The overall results suggested that Bcr1 is required for both *ALS3* and *HWP1* expression during OPC, a functional relationship similar to that described *in vitro* (35).

Here we have looked at gene expression during OPC to understand the basis for the *bcr1Δ/Δ* mutant phenotype. Our study has been aided by new technology, the nanoString nCounter, which has sensitivity on par with that of real-time RT-PCR yet is capable of profiling over 100 genes per sample (17). We expected that this assay platform may be useful for infected tissue samples, because it enables selective assay of pathogen gene expression despite the presence of abundant host RNA. This issue has previously been recognized as a major impediment to analysis of infection-related gene expression (2). Our findings indicate that many genes that are Bcr1 dependent *in vitro* are still expressed in the mutant during infection. These results suggest that a Bcr1 bypass pathway may function *in vivo* to activate shared target genes. In a broader context, our results emphasize the value of mutant gene expression profiling *in vivo* as a way to understand gene function during infection.

MATERIALS AND METHODS

Media and strain construction. *C. albicans* strains were grown on YPD (2% Bacto peptone, 2% dextrose,1% yeast extract) or in Spider medium (1% nutrient broth [BD Difco], 1% D-mannitol [Sigma], 0.2% K$_2$HPO$_4$ [Sigma]). *C. albicans* strains used in this study are listed in Table 1.

Mouse model of oropharyngeal candidiasis. To assess the virulence of the various strains of *C. albicans*, the OPC mouse model was used (6, 37). This study was approved by the Animal Use Committee of the Los Angeles Biomedical Institute in compliance with NIH guidelines for the ethical treatment of animals. Male BALB/c mice (National Cancer Institute) weighing approximately 20 g were immunosuppressed with cortisone acetate (Sigma-Aldrich) at a dose of 225 mg/kg of body weight administered subcutaneously on days −1, +1, and + 3 relative to the day of infection. To induce OPC, the mice were anesthetized with xylazine and ketamine (both from Phoenix pharmaceuticals) administered intraperitoneally. Next, calcium alginate urethral swabs (Type 4 Calgiswab; Puritan Medical Products Company LLC) were saturated with *C. albicans* by placing them in Hanks balanced salt solution (HBSS) containing 10^6 organisms per ml. The saturated swabs were placed sublingually in the anesthetized mice for 75 min. Each strain of *C. albicans* was inoculated into 7 to 9 mice per experiment. The virulence of the *bcr1Δ/Δ* mutant was compared to those of the wild-type and *bcr1Δ/Δ::BCR1*-complemented strains in multiple experiments involving a total of 20 to 30 mice per strain. The virulence of the *als3* and *hwp1* mutants was tested in single experiments. After the mice recovered from anesthesia, they were given food and water *ad libitum*. The mice were sacrificed after either 1 or 5 days of infection, after which their tongues and adjacent sublingual tissue were excised. The tissue was weighed, homogenized, and quantitatively cultured on Sabouraud dextrose agar containing chloramphenicol. Differences in oral fungal burden among mice infected with different strains were analyzed using the Wilcoxon rank sum test.

RNA isolation. Immediately after being harvested, the tongues were cut into small pieces on ice and then immersed in RNAlater (Ambion). They were stored in this solution at 4°C overnight and then at −20°C. The frozen samples were then homogenized in trireagent (Ambion) using a Fastprep FP120 (Bio101) instrument at setting 4 using ceramic bead no. 2. The RNA was then isolated using the Ribopure kit (Ambion) following the manufacturer's instruction.

NanoString analysis of gene expression. Samples containing 100 ng of *C. albicans* total RNA (from samples grown in Spider media) or 5 to 10 μg of *C. albicans*-infected mouse tissue RNA were mixed with custom-designed probe code set and incubated at 65°C overnight (12 to 18 h). The hybridized samples were processed on a nanoString prep station using the manufacturer's default program. The resultant cartridges were then transferred to the nanoString digital analyzer and scanned for 600 fields per sample. The raw counts were first adjusted for technical variability using the positive and negative controls of irrelevant RNA sequences included in the code set. The technically adjusted counts were then normalized for total input *C. albicans* RNA using the housekeeping gene *TDH3*. The normalized counts were used to compare gene expression levels among different samples.
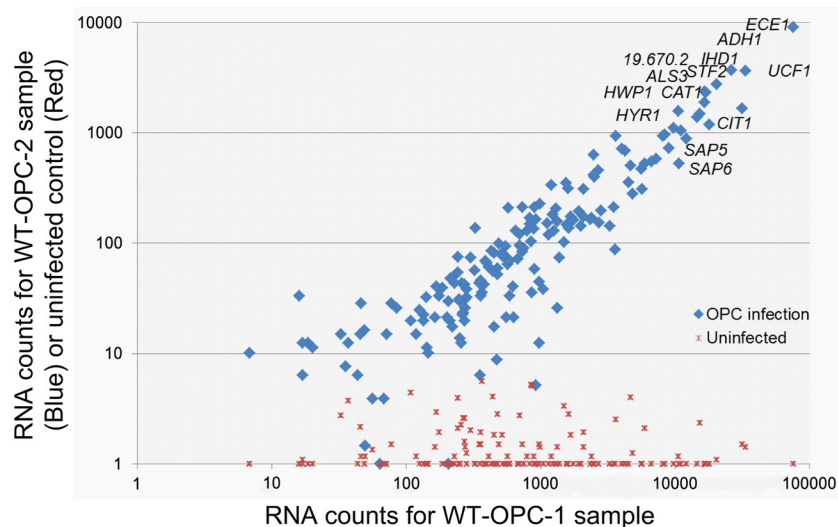
FIG 1 Comparison of *C. albicans* RNA levels in mouse tongue samples. RNA was isolated from infected tongues (WT-OPC-1 and WT-OPC-2) or an uninfected tongue and used for RNA level measurements by nanoString analysis. In total, the RNA levels for 134 *C. albicans* genes were determined. The scatter plot presents each RNA level for one infected tongue (WT-OPC-1) compared to either an independent infected tongue (WT-OPC-2, blue data points) or the uninfected tongue (red data points). These samples all had background subtracted but were not normalized to the level of *TDH3* (control) RNA. *C. albicans* strain DAY185 was used for infections.

NanoString data analysis. We used MultiExperimentViewer (MeV v4.6.2) to cluster data sets. The normalized data sets were used to determine if the expression level of a gene in a strain under a particular condition was significantly different from that of another. Three determinations for a gene in the *bcr1*Δ/Δ mutant grown in Spider medium were divided by the average determination for a gene in the wild-type strain grown in Spider. Three determinations for a gene in the *bcr1*Δ/Δ mutant in OPC infection (3 independent biological samples) were divided by the average determination for a gene in the wild-type strain in OPC infection (3 independent biological samples). The normalized data (fold change) were log$_2$ transformed and subjected to averaging linkage clustering based on Manhattan distance.

RESULTS AND DISCUSSION

Gene expression during OPC infection. To obtain insight into *C. albicans* gene expression during OPC infection, we employed the nanoString nCounter system (17). This assay counts specific RNA molecules directly in a sample through bar-coded probe readouts. Because this technology is not genome-wide, it is critical to choose informative genes for expression measurements. We chose 134 genes for assays based upon many published microarray data sets and our nanoString *in vitro* data sets. Specifically, we selected representative genes that are upregulated in response to conditions that included azole treatment (26), biofilm formation *in vitro* (16), biofilm formation *in vivo* (32), general stress (13), hyphal growth (4), hypoxia (46), iron limitation (43), kidney infection (49), oxidative stress (12), and caspofungin treatment (3) and during specific cell cycle phases (7). We also included genes that were highly responsive to adherence regulators (14). We included a control gene, *TDH3*, for normalization among samples and backup control genes for high expression, *ACT1*, and for low-to-moderate expression, *ARP3* and *YRA1*. These control genes varied little in numerous microarray studies and *in vitro* nanoString studies from our lab (see Data File S1, Gene Selection Tab, in the supplemental material). Thus, our panel of genes presented the opportunity to relate anticipated findings to well-studied pathways and re-

sponses. In addition, we had likely enriched for reporter genes that were regulated at the level of RNA accumulation.

Gene expression was assayed during *in vivo* OPC infection using infected mouse tongue tissue as a source of RNA. We compared RNA levels detected by our probes among tissue samples from three animals (Fig. 1; see also Data File S1 in the supplemental material). The nanoString platform detected C. *albicans* RNA transcript levels from *in vivo* samples even though the total RNA sample contained less than 0.1% *Candida* RNA, as estimated from *C. albicans TDH3* RNA levels. The relative expression levels of our reporter genes were highly reproducible. For example, for the two *in vivo* infection samples presented in Fig. 1, there was a 10-fold difference in the quantity of *C. albicans* RNA, as seen by comparison of the *x* and *y* axes. Nonetheless, signals were well above background. For example, the ratio of median signal counts to maximum background counts was roughly 50 (400 counts/8 counts). Most importantly, the relative expression levels among the genes were consistent in the two samples (Fig. 1). Similar agreement was evident among other comparisons of biological replicates (see Fig. S1 and S2 in the supplemental material).

The genes most highly expressed in murine OPC samples (Fig. 1) included *ALS3*, *HWP1*, and *ECE1*, in keeping with prior studies of piglet OPC infection and RHE infection (50, 54), and *SAP5* and *SAP6*, as expected from studies of patient samples (30). These results, along with high-level expression of other hyphal genes (*IHD1* and *HYR1*), fit well with the observation that hyphae are prominent in infected tissue samples (23). Genes repressed by Hap43 or high iron levels (5, 20, 43) were also highly expressed (*orf19.670.2*, *UCF1*, and *CAT1*), which is consistent with the finding that the iron-repressed *CFL2* and *FRE4* transcripts are highly expressed in human patient samples (54). These results suggest that iron is limiting during OPC infection in this murine model.

We also sought to define gene expression relationships between infection and *in vitro* growth. To do so, we compared OPC infection RNA measurements to previous RNA measurements on
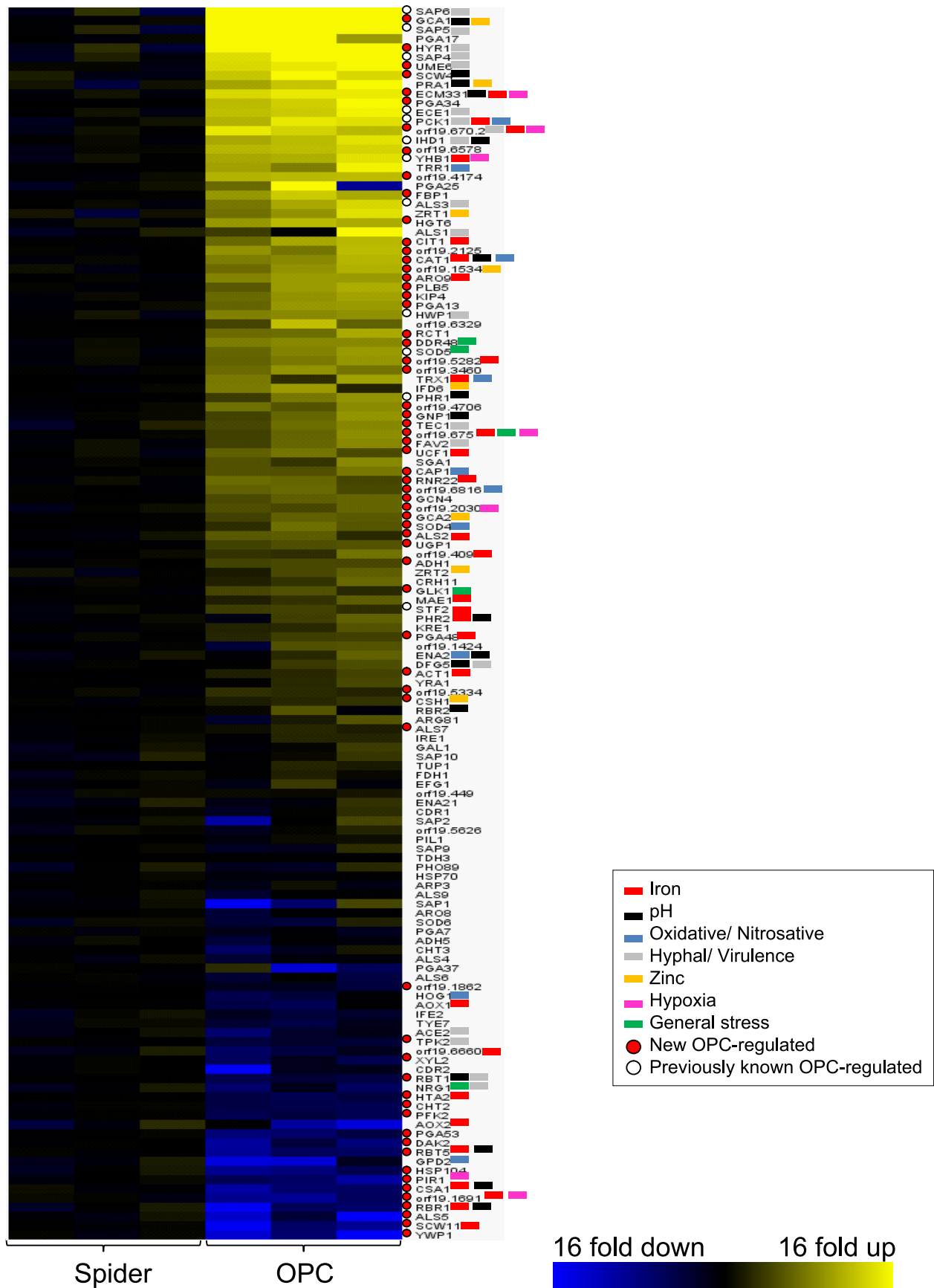
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TABLE 2 Classes of genes that are upregulated during OPC infection

Response pathway[a]	Representative genes[b]
General stress	*DDR48, orf19.675, SOD5[c]*
Alkaline pH	*CAT1, ECM331, GCA1, GNP1, IHD1[c], PHR1[c], PHR2, PRA1[d], SCW4*
Oxidative stress	*CAP1, CAT1, orf19.6816, TRR1[d], TRX1[d], SOD4*
Nitrosative stress	*TRR1[d], YHB1[c], CAT1*
Iron limitation	*ALS2, ARO9, CAT1, CIT1, ECM331, orf19.409[d], orf19.5282, orf19.670.2, orf19.675, PCK1[c], PGA48, PHR2, RNR22, STF2, TRX1, UCF1, YHB1[c]*
Zinc limitation	*CSH1, GCA1, GCA2, IFD6[d], orf19.1534, PRA1[d], ZRT1[d], ZRT2[d]*
Hypoxia	*ECM331, orf19.2030, orf19.670.2, orf19.675, orf19.670.2, PCK1[c]*
Hyphal and virulence	*ALS1[d], ALS3[c], ECE1[c], FAV2, HWP1[c], HYR1, IHD1[c], orf19.670.2, PCK1[c], SAP4[c], SAP5[c], SAP6[c], TEC1, UME6*
Other	*ADH1, ALS7, FBP1, GCN4, HGT6, KIP4, orf19.2125, orf19.3460, orf19.4174, orf19.4706, orf19.6329[d], orf19.6578, PGA13, PGA17[d], PGA25[d], PGA34, PLB5, RCT1, SGA1, UGP1*

[a] Response pathways, defined by *in vitro* studies, are pathways whose activation results in upregulation of the genes listed.
[b] The *C. albicans* genes listed were upregulated in OPC infection samples compared to *in vitro* growth in Spider medium at 37°C. Note that some genes are activated by multiple pathways and thus may be listed more than once.
[c] Gene identified as OPC-upregulated gene in prior studies.
[d] Gene trended toward upregulation but had a *t* test *P* value of <0.1 and >0.05. All other genes listed displayed significant upregulation, reflecting a *t* test *P* value of <0.05.

the nanoString platform (14) during growth in Spider medium at 37°C (Fig. 2; see Data File S1 in the supplemental material). Assays of gene expression levels in YPD medium at 30°C were also examined (see Fig. S2 in the supplemental material), because this growth condition has been used for comparison in some previous studies. As expected, the gene expression profile of cells grown in YPD at 30°C was vastly different from that of cells grown in Spider at 37°C or in OPC infection (see Fig. S2 in the supplemental material). We used Spider medium for further comparisons here because it induces the formation of hyphae, which are also prominent in OPC samples.

Gene expression during OPC infection was different from that in Spider medium; about one-half of the genes assayed were expressed at different levels under the two conditions (Fig. 2; Table 2; *P* values are shown in Data File S1 in the supplemental material). The most striking feature of OPC gene expression was that representatives of virtually every stress response pathway were upregulated (Fig. 2). Noteworthy were oxidative stress response genes. The oxidative stress response is not observed in RHE infection models (54) and probably reflects interaction of *C. albicans* with residual macrophages and neutrophils that are present in infected tissue, despite immunosuppression. Several hypoxic stress genes were upregulated, likely a reflection of oxygen limitation in cells that invade tissues. Upregulation of this gene class was also

not evident in prior RHE infection studies (54). Many iron and zinc limitation genes were also upregulated, as expected if these nutrients are limiting during infection. Surprisingly, despite the fact that our comparison of OPC samples was to hyphal cells, many hyphal genes were expressed at significantly higher levels in the OPC samples. Induction of these genes during OPC infection was greater than in many serum-containing media as well in our preliminary studies (see Data File S2 in the supplemental material). Finally, a useful outcome of gene expression analysis is the identification of new candidate genes that may have functional roles during infection. Although our probe selection was biased toward genes of known function, we note that several OPC-upregulated genes are uncharacterized (*orf19.4174, orf19.2125, orf19.4706, orf19.3460,* and *orf19.6329*) and may be interesting for future analysis. All told, our findings argue that the success of *C. albicans* as an oral pathogen may reflect its ability to mount multiple stress responses. Conversely, if we view *C. albicans* as a biosensor, then we infer that the oral cavity is an inhospitable environment, even after immunosuppression.

It seemed possible that our experiments might only verify the microarray-based identification of *C. albicans* genes upregulated in RHE infection and OPC patient samples (54). Indeed, we did verify upregulation during OPC infection of the highly expressed genes *ALS3, ECE1, HWP1, IHD1, PCK1, PHR1, SAP5, SOD5, STF2,* and *YHB1.* However, we also defined 62 new genes whose RNA levels are regulated during OPC. If YPD 30°C cell samples are used as the comparator, this number increases to 83 new OPC-regulated genes. These findings probably reflect the sensitivity of the nanoString, because the previously known OPC-upregulated genes all have RNA levels above the median in our experiments.

Bcr1-responsive gene expression *in vivo*. In order to understand Bcr1-dependent gene expression *in vivo*, we analyzed RNA from tongues of three mice that had been infected with a *bcr1Δ/Δ* strain. For comparison to *in vitro*-grown cells, we used our recently published data set (14) of *bcr1Δ/Δ* cells grown in Spider medium at 37°C. *In vivo* and *in vitro* measurements were normalized for each gene to the mean wild-type expression levels under comparable growth conditions (Fig. 3). The data for the *bcr1Δ/Δ* strain were highly reproducible among biological replicates (Fig. 3; see Fig. S1 in the supplemental material). The data set for *in vitro*-grown cells correlated well for previously reported Bcr1-dependent genes (including *ALS1, ALS3, HWP1, CHT2, ECE1,* and *RBT5*) from *in vitro* RNA microarrays (35) and RNAseq studies (34). Several additional Bcr1-dependent genes (including *SOD5, IFE2, PGA7,* and *PHO89*) were detected only in nanoString (14) and RNAseq (34) data sets. These results indicate that Bcr1-responsive genes defined under *in vitro* growth conditions are highly reproducible, extending across different detection methods and laboratories.

In contrast, the spectrum of Bcr1-responsive genes from *in vivo* OPC infection samples was quite different from that *in vitro* (Fig. 3; see Data File S1 in the supplemental material). A core set of genes had significantly altered expression in the mutant both *in*

FIG 2 Relative *C. albicans* RNA levels from *in vitro* growth and OPC infection. NanoString measurements were conducted on three independent cultures of *C. albicans* grown *in vitro* (labeled "Spider") or on three infected mouse tongues ("OPC"). RNA levels for each gene were normalized to internal control *TDH3* RNA levels and are presented as ratios to the mean RNA level for each gene in the *in vitro* samples. In the heat map columns, upregulation is represented by yellow, downregulation is represented by blue, and the magnitude of regulation is reflected by color saturation. Known regulatory pathways that govern each gene are presented in the color bars to the right of each gene name. *C. albicans* strain DAY185 was used for all assays.
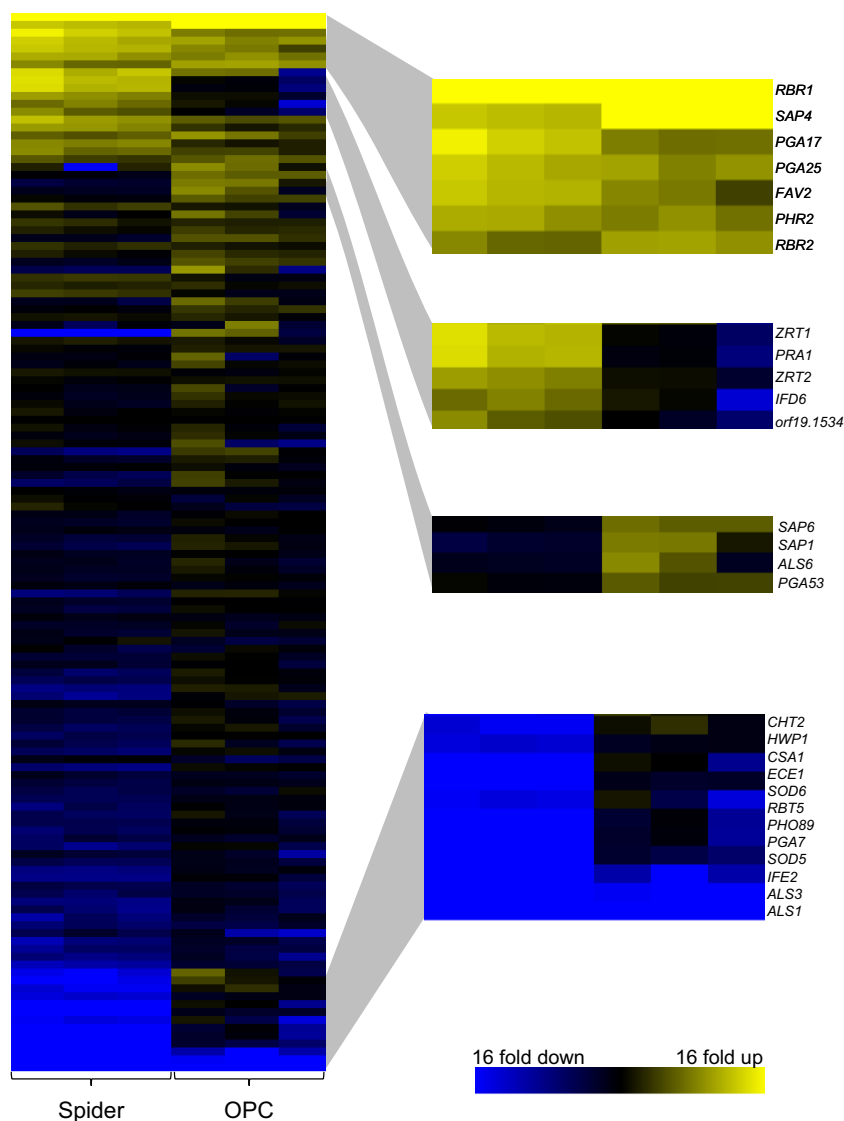
FIG 3 Relative *bcr1Δ/Δ* mutant RNA levels from *in vitro* growth and OPC infection. NanoString measurements were conducted on *C. albicans* strain CJN702 (*bcr1Δ/Δ*), using three independent cultures grown *in vitro* (labeled "Spider") or on three infected mouse tongues ("OPC"). RNA levels for each gene were normalized to internal control *TDH3* RNA levels and are presented as ratios to the mean RNA level for each gene in samples from wild-type strain DAY185. Upregulation is represented by yellow; downregulation is represented by blue; the magnitude of regulation is reflected by color saturation.

vivo and *in vitro*, including downregulated genes *ALS1*, *ALS3*, and *IFE2* and upregulated genes *RBR1*, *SAP4*, *PGA17*, and *PGA25*. Some upregulated genes are repressed by *RIM101* (*PHR2*, *RBR1*, and *RBR2* [28]). This response may reflect the facts that Bcr1 activates expression of *RIM8* (34) and Rim8 is an upstream member of the Rim101 pathway (9). However, most striking were the many examples of genes that were Bcr1 responsive only *in vitro* or *in vivo*. Prominent in this class were many hyphal genes, including *HWP1* and *ECE1* (Fig. 3). Our results indicate that environmental conditions can alter the spectrum of Bcr1-responsive genes considerably. Although there are previous reports of other transcription factors whose function is bypassed *in vivo* for specific target genes (38, 50; reviewed in reference 24), the possibility that most targets of a transcription factor are altered *in vivo* has not previously been investigated. Our findings are in keeping with Kumamoto's suggestion that several of the known hyphal gene reg-

ulators may have overlapping functions *in vivo* (24), though recent discoveries have expanded the number of hyphal gene regulators dramatically (14, 34). One might imagine that the differential regulation separates direct and indirect Bcr1 target genes: the more stringent *in vivo* growth conditions might result in only direct Bcr1 target genes showing Bcr1-dependent expression. However, based on genome-wide chromatin immunoprecipitation (ChIP) data from Nobile et al. (34), this hypothesis seems incorrect. For example, *IFE2* and *ALS3* are indirect Bcr1 target genes (regulated by Bcr1 but not bound by Bcr1), yet these are Bcr1 dependent *in vitro* and *in vivo*. In contrast, *RBT5* and *CHT2* are direct Bcr1 targets yet show Bcr1-dependent expression only *in vitro*. Given the extensive upregulation of *C. albicans* stress response pathways during OPC infection, perhaps one or more stress response pathways may override Bcr1 dependence for many target genes.

Functional analysis of *BCR1*, *ALS3*, and *HWP1* in vivo. We
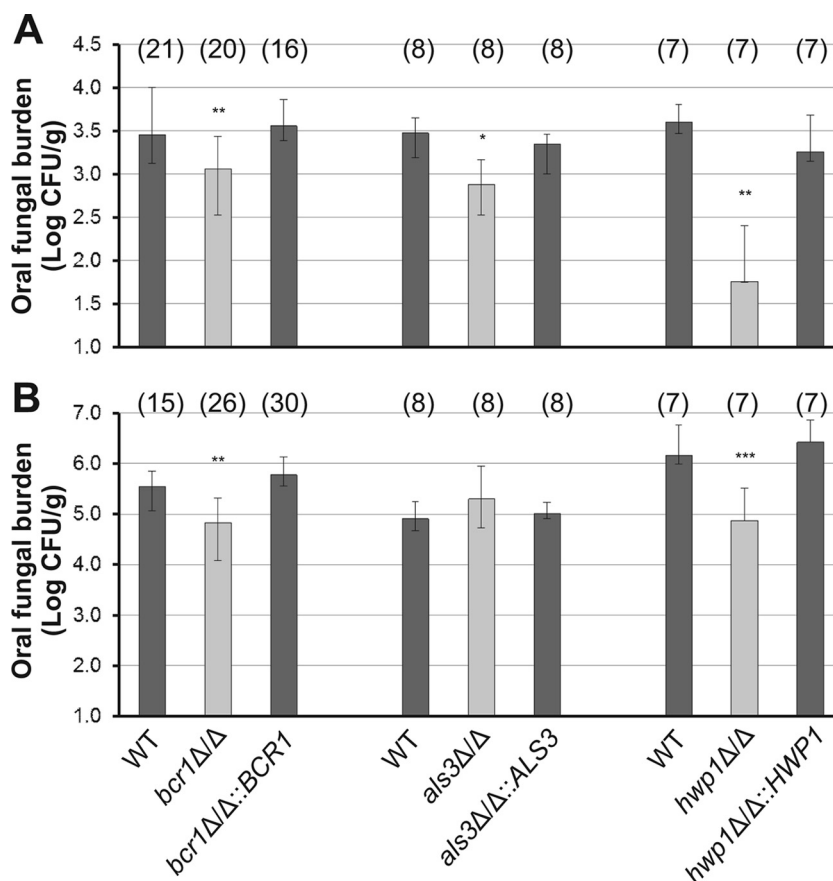
FIG 4 Contribution of Bcr1, Als3, and Hwp1 to virulence in OPC infection. Immunosuppressed mice were inoculated with *C. albicans* strains with the genotypes indicated. After 1 day (A) or 5 days (B), mice were sacrificed, and the oral fungal burdens were calculated. Results are medians ± interquartile ranges. Statistical analysis was performed relative to wild-type fungal burdens by the Wilcoxon rank sum test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.0001$. The numbers of animals for each determination are given in parentheses across the top of each graph.

selected the adhesin genes *ALS3* and *HWP1* for functional analysis, with the specific goal of assessing virulence potential during OPC infection of null mutants under uniform assay conditions. *HWP1* has been implicated in OPC based on its overexpression (11) and on *in vivo* null mutant assays in a transgenic mouse model (44). A role for *ALS3* in OPC is uncertain (11). We first verified that Bcr1 is required for OPC infection. We observed that a *bcr1Δ/Δ* mutant produced a significantly lower fungal burden than the wild-type strain at days 1 and 5 of infection (Fig. 4). Parallel assays indicated that Als3 is required only early in infection: an *als3Δ/Δ* mutant had significantly lower fungal burden than the wild type at day 1 of infection but had no defect at day 5 (Fig. 4). The overall role of Als3 seemed modest. Hwp1 has a prominent role at all times: an *hwp1Δ/Δ* strain yielded significantly reduced fungal burden at days 1 and 5 of infection (Fig. 4). These results verify that Bcr1, Als3, and Hwp1 have roles in OPC infection despite their altered relationship based on expression analysis above. In addition, the fact that an *als3Δ/Δ* strain has a milder and more transient OPC defect than a *bcr1Δ/Δ* strain helps to explain why overexpression of *ALS3* does not fully rescue the *bcr1Δ/Δ* phenotype (11): there must be additional Bcr1-dependent genes that contribute to the *bcr1Δ/Δ* OPC defect.

Our gene expression data provide only a limited view of OPC infection, but they improve our understanding of OPC in several

ways. The most important point is that infecting cells express targets of numerous stress response and nutrient limitation pathways. Our findings extend what was known from pioneering microarray profiling studies of RHE and patient samples (54). Thus, the versatility of this pathogen may reflect its ability to endure multiple stresses. A second important point is that many new genes that are highly expressed or highly upregulated during OPC infection have been identified. While many of these genes have functional roles that can be deduced from homology, many others do not, and they are thus candidates for future functional studies. Finally, our results suggest a new twist on the idea that *C. albicans* transcriptional regulatory circuits have been rewired (25). It is generally appreciated that evolutionary forces have reshaped the features of transcriptional circuits in *C. albicans*, compared to *Saccharomyces cerevisiae*. Our studies, as well as those of Kumamoto and colleagues (50), underscore the fact that transcriptional circuits we study *in vitro* may also be reshaped during infection. We believe that an understanding of gene expression during growth *in vivo* is critical to validate *in vitro* studies and to define and prioritize the voids in our understanding.

ACKNOWLEDGMENTS

REFERENCES

1. **Bensadoun RJ, Patton LL, Lalla RV, Epstein JB.** 2011. Oropharyngeal candidiasis in head and neck cancer patients treated with radiation: update 2011. Support Care Cancer **19**:737–744.
2. **Brown AJ, Odds FC, Gow NA.** 2007. Infection-related gene expression in Candida albicans. Curr. Opin. Microbiol. **10**:307–313.
3. **Bruno VM, et al.** 2006. Control of the C. albicans cell wall damage response by transcriptional regulator Cas5. PLoS Pathog. **2**:e21. doi: 10.1371/journal.ppat.0020021.
4. **Cao F, et al.** 2006. The Flo8 transcription factor is essential for hyphal development and virulence in Candida albicans. Mol. Biol. Cell **17**:295–307.
5. **Chen C, Pande K, French SD, Tuch BB, Noble SM.** 2011. An iron homeostasis regulatory circuit with reciprocal roles in Candida albicans commensalism and pathogenesis. Cell Host Microbe **10**:118–135.
6. **Chiang LY, et al.** 2007. Candida albicans protein kinase CK2 governs virulence during oropharyngeal candidiasis. Cell. Microbiol. **9**:233–245.
7. **Cote P, Hogues H, Whiteway M.** 2009. Transcriptional analysis of the Candida albicans cell cycle. Mol. Biol. Cell **20**:3363–3373.
8. **Davis D, Edwards JE, Jr, Mitchell AP, Ibrahim AS.** 2000. Candida albicans RIM101 pH response pathway is required for host-pathogen interactions. Infect. Immun. **68**:5953–5959.
9. **Davis DA.** 2009. How human pathogenic fungi sense and adapt to pH: the link to virulence. Curr. Opin. Microbiol. **12**:365–370.
10. **Dongari-Bagtzoglou A, Kashleva H, Dwivedi P, Diaz P, Vasilakos J.** 2009. Characterization of mucosal Candida albicans biofilms. PLoS One **4**:e7967. doi:10.1371/journal.pone.0007967.
11. **Dwivedi P, et al.** 2011. Role of Bcr1-activated genes Hwp1 and Hyr1 in Candida albicans oral mucosal biofilms and neutrophil evasion. PLoS One **6**:e16218. doi:10.1371/journal.pone.0016218.
12. **Enjalbert B, Nantel A, Whiteway M.** 2003. Stress-induced gene expression in Candida albicans: absence of a general stress response. Mol. Biol. Cell **14**:1460–1467.
13. **Enjalbert B, et al.** 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen Candida albicans. Mol. Biol. Cell **17**:1018–1032.
14. **Finkel JS, et al.** 2012. Portrait of Candida albicans adherence regulators. PLoS Pathog. **8**:e1002525. doi:10.1371/journal.ppat.1002525.
15. **Ganguly S, Mitchell AP.** 2011. Mucosal biofilms of Candida albicans. Curr. Opin. Microbiol. **14**:380–385.
16. **Garcia-Sanchez S, et al.** 2004. Candida albicans biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot. Cell **3**:536–545.
17. **Geiss GK, et al.** 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat. Biotechnol. **26**:317–325.
18. **Gow NA, van de Veerdonk FL, Brown AJ, Netea MG.** 2011. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat. Rev. Microbiol. **10**:112–122.
19. **Harriott MM, Lilly EA, Rodriguez TE, Fidel PL, Jr, Noverr MC.** 2010. Candida albicans forms biofilms on the vaginal mucosa. Microbiology **156**:3635–3644.
20. **Hsu PC, Yang CY, Lan CY.** 2011. *Candida albicans* Hap43 is a repressor induced under low-iron conditions and is essential for iron-responsive transcriptional regulation and virulence. Eukaryot. Cell **10**:207–225.
21. **Hube B.** 2006. Infection-associated genes of Candida albicans. Future Microbiol. **1**:209–218.
22. **Hughes TR, et al.** 2000. Functional discovery via a compendium of expression profiles. Cell **102**:109–126.
23. **Kamai Y, Kubota M, Hosokawa T, Fukuoka T, Filler SG.** 2001. New model of oropharyngeal candidiasis in mice. Antimicrob. Agents Chemother. **45**:3195–3197.
24. **Kumamoto CA.** 2008. Niche-specific gene expression during C. albicans infection. Curr. Opin. Microbiol. **11**:325–330.
25. **Lavoie H, et al.** 2010. Evolutionary tinkering with conserved components of a transcriptional regulatory network. PLoS Biol. **8**:e1000329. doi: 10.1371/journal.pbio.1000329.
26. **Liu TT, et al.** 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in Candida albicans. Antimicrob. Agents Chemother. **49**:2226–2236.
27. **Lo HJ, et al.** 1997. Nonfilamentous C. albicans mutants are avirulent. Cell **90**:939–949.
28. **Lotz H, Sohn K, Brunner H, Muhlschlegel FA, Rupp S.** 2004. RBR1, a novel pH-regulated cell wall gene of Candida albicans, is repressed by RIM101 and activated by NRG1. Eukaryot. Cell **3**:776–784.
29. **Martin R, Wachtler B, Schaller M, Wilson D, Hube B.** 2011. Host-pathogen interactions and virulence-associated genes during Candida albicans oral infections. Int. J. Med. Microbiol. **301**:417–422.
30. **Naglik JR, et al.** 2008. Quantitative expression of the Candida albicans secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. Microbiology **154**:3266–3280.
31. **Nailis H, et al.** 2010. Real-time PCR expression profiling of genes encoding potential virulence factors in Candida albicans biofilms: identification of model-dependent and -independent gene expression. BMC Microbiol. **10**:114. doi:10.1186/1471-2180-10-114.
32. **Nett JE, Lepak AJ, Marchillo K, Andes DR.** 2009. Time course global gene expression analysis of an in vivo Candida biofilm. J. Infect. Dis. **200**:307–313.
33. **Nobile CJ, et al.** 2006. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. **2**:e63. doi: 10.1371/journal.ppat.0020063.
34. **Nobile CJ, et al.** 2012. A recently evolved transcriptional network controls biofilm development in Candida albicans. Cell **148**:126–138.
35. **Nobile CJ, Mitchell AP.** 2005. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr. Biol. **15**:1150–1155.
36. **Park H, et al.** 2009. Transcriptional responses of *Candida albicans* to epithelial and endothelial cells. Eukaryot. Cell **8**:1498–1510.
37. **Park H, et al.** 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell. Microbiol. **7**:499–510.
38. **Rosenbach A, Dignard D, Pierce JV, Whiteway M, Kumamoto CA.** 2010. Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. Eukaryot. Cell **9**:1075–1086.
39. **Samaranayake LP, Keung Leung W, Jin L.** 2009. Oral mucosal fungal infections. Periodontol. 2000 **49**:39–59.
40. **Sanchez AA, et al.** 2004. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. Infect. Immun. **72**:598–601.
41. **Sangeorzan JA, et al.** 1994. Epidemiology of oral candidiasis in HIV-infected patients: colonization, infection, treatment, and emergence of fluconazole resistance. Am. J. Med. **97**:339–346.
42. **Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot. Cell **2**:1053–1060.
43. **Singh RP, Prasad HK, Sinha I, Agarwal N, Natarajan K.** 2011. Cap2-HAP complex is a critical transcriptional regulator that has dual but contrasting roles in regulation of iron homeostasis in Candida albicans. J. Biol. Chem. **286**:25154–25170.
44. **Sundstrom P, Balish E, Allen CM.** 2002. Essential role of the Candida albicans transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. J. Infect. Dis. **185**:521–530.
45. **Sundstrom P, Cutler JE, Staab JF.** 2002. Reevaluation of the role of HWP1 in systemic candidiasis by use of *Candida albicans* strains with selectable marker URA3 targeted to the ENO1 locus. Infect. Immun. **70**:3281–3283.
46. **Synnott JM, Guida A, Mulhern-Haughey S, Higgins DG, Butler G.** 2010. Regulation of the hypoxic response in *Candida albicans*. Eukaryot. Cell **9**:1734–1746.
47. **Thewes S, et al.** 2007. In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive Candida albicans isolates identifies genes associated with tissue invasion. Mol. Microbiol. **63**:1606–1628.
48. **Thompson GR, III, et al.** 2010. Oropharyngeal candidiasis in the era of antiretroviral therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. **109**:488–495.
49. **Walker LA, et al.** 2009. Genome-wide analysis of Candida albicans gene expression patterns during infection of the mammalian kidney. Fungal Genet. Biol. **46**:210–219.
50. **White SJ, et al.** 2007. Self-regulation of Candida albicans population size

during GI colonization. PLoS Pathog. **3**:e184. doi:10.1371/journal.ppat.0030184.

51. **Willis AM, et al.** 1999. Oral candidal carriage and infection in insulin-treated diabetic patients. Diabet. Med. **16**:675–679.

52. **Wilson D, et al.** 2009. Identifying infection-associated genes of Candida albicans in the postgenomic era. FEMS Yeast Res. **9**:688–700.

53. **Wilson RB, Davis D, Mitchell AP.** 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. **181**:1868–1874.

54. **Zakikhany K, et al.** 2007. In vivo transcript profiling of Candida albicans identifies a gene essential for interepithelial dissemination. Cell. Microbiol. **9**:2938–2954.